5-Lipoxygenase and 5-Lipoxygenase-activating Protein Are Localized in the Nuclear Envelope of Activated Human Leukocytes

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Summary

The intracellular distribution of the enzyme 5-lipoxygenase (5-LO) and 5-lipoxygenase–activating protein (FLAP) in resting and ionophore-activated human leukocytes has been determined using immuno-electronmicroscopic labeling of ultrathin frozen sections and subcellular fractionation techniques. 5-LO is a 78-kD protein that catalyzes the conversion of arachidonic acid to leukotrienes. FLAP is an 18-kD membrane bound protein that is essential for leukotriene synthesis in cells. In response to ionophore stimulation, 5-LO translocates from a soluble to a sedimentable fraction of cell homogenates. In activated leukocytes, both FLAP and 5-LO were localized in the lumen of the nuclear envelope. Neither protein could be detected in any other cell compartment or along the plasma membrane. In resting cells, the FLAP distribution was identical to that observed in activated cells. In addition, subcellular fractionation techniques showed >83% of immunoblot–detectable FLAP protein and ∼64% of the FLAP ligand binding activity was found in the nuclear membrane fraction. A fractionation control demonstrated that a plasma membrane marker, detected by a monoclonal antibody PMN13F6, was not detectable in the nuclear membrane fraction. In contrast to FLAP, 5-LO in resting cells could not be visualized along the nuclear envelope. Except for weak labeling of the euchromatin region of the nucleus, 5-LO could not be readily detected in any other cellular compartment. These results demonstrate that the nuclear envelope is the intracellular site at which 5-LO and FLAP act to metabolize arachidonic acid, and that ionophore activation of neutrophils and monocytes results in the translocation of 5-LO from a nonsedimentable location to the nuclear envelope.

Leukotrienes are products of arachidonic acid metabolism that are produced by leukocytes and that have a variety of effects on the immune system (1). The enzyme 5-lipoxygenase (5-LO,[1] EC 1.13.11.12) catalyzes two key steps in the leukotriene biosynthetic pathway (2, 3). These steps are the oxygenation of arachidonate to 5-(*S*)-hydroperoxy-6,8, 11,14-eicosatetraenoic acid (5-HPETE), followed by its dehydrogenation, which results in the formation of the unstable epoxide LTA_4. In neutrophils the enzyme LTA_4 hydrolase then converts LTA_4 to LTB_4, which is a potent chemotactic and activating factor for neutrophils and eosinophils (4–6). In eosinophils and mast cells, LTA_4 can also be converted to LTC_4, LTD_4, and LTE_4, all of which stimulate contraction of vascular and bronchial smooth muscle (7, 8), resulting in effects on local circulation and bronchoconstriction. These potent biological effects implicate leukotrienes in a number of hypersensitivity and inflammatory disorders, including asthma and inflammatory bowel disease (9–12).

The enzyme 5-LO has been purified from a number of mammalian leukocyte sources (2, 3, 13). In every case the purified enzyme was activated by calcium and ATP. It was observed that when subcellular fractions of unstimulated human leukocytes were examined for 5-LO distribution, >90% of the immunodetectable enzyme was found in the 100,000-*g* supernatant fraction. However, in response to challenge with the calcium ionophore A23187, 5-LO translocated from a supernatant fraction to a sedimentable fraction (2, 14), strongly suggesting that membrane association plays a key role in the in vivo regulation of 5-LO activity.

[1] *Abbreviations used in this paper:* FLAP, 5-lipoxygenase-activating protein; GAM, goat anti-mouse IgG; GAR, goat anti-rabbit IgG; 5-HPETE, 5-(*S*)-hydroperoxy-6,8,11,14-eicosatetraenoic acid; 5-LO, 5-lipoxygenase; LSM, lymphocyte separation medium; PLP, paraformaldehyde-lysine-periodate.

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Studies with the leukotriene synthesis inhibitor MK-886 have led to the identification of an 18-kD membrane–bound protein, 5-lipoxygenase–activating protein (FLAP), which has an essential role in the regulation of cellular 5-LO-activity (15). Observation of the cell and tissue distribution of 5-LO and FLAP has indicated that both of these proteins are present in all leukotriene producing cells analyzed thus far (16). Further evidence demonstrating that FLAP is essential for 5-LO activity in intact cells comes from transfection experiments that showed that cells lacking either protein must be cotransfected with cDNAs for both in order to produce leukotrienes in response to calcium ionophore stimulation (17).

At present there are no reports describing the ultrastructural localization of FLAP and conflicting reports describing the localization of 5-LO. Ultrastructural studies have reported that in leukocytes 5-LO was localized in the cytoplasm (18), whereas it was associated with the nuclear envelope in the corpus luteum (19) and pancreatic acinar cells (20). Subcellular fractionation studies have found that in human polymorphonuclear leukocytes 5-LO was found in the specific granules (21). None of these studies addressed the effect of activation of leukotriene synthesis on the subcellular distribution of 5-LO. This report will describe a series of immunoelectronmicroscopic observations and subcellular fraction experiments designed to answer the following questions: (*a*) what is the subcellular distribution of 5-LO and FLAP in human leukocytes and (*b*) what is the ultrastructural correlate of the 5-LO translocation event observed after ionophore activation?

Materials and Methods

Materials. Rabbit polyclonal antiserum to highly purified 5-LO (22) was prepared by Hazelton Research Laboratories (Denver, PA) by injection of 200 μg of purified 5-LO followed by two boosts of 100 μg each at 1-mo intervals. The rabbit was then bled biweekly for 6 mo. Immunoblot analysis showed that all bleeds recognized a 78-kD protein present in supernatant fractions of human leukocytes. The preparation of rabbit anti-peptide antiserum specific for amino acids 41–52 of FLAP was as previously described (23). IgG from these antisera was isolated by chromatography over Protein A (Pierce, Rockford, IL). Preimmune IgG was also prepared from these rabbits. Mouse polyclonal antiserum specific for human 5-LO was obtained by immunizing mice with purified human 5-LO (24). H52, a hybridoma producing a monoclonal antibody specific for CD18 (25), was obtained from the Developmental Studies Hybridoma Bank (Baltimore, MD) and PMN13F6 ascites fluid containing a monoclonal antibody specific for a PMN plasma membrane protein (26, 27) was purchased from Sigma Chemical Co. (St. Louis, MO). Dextran T500 was from Pharmacia AB (Uppsala, Sweden), Dulbecco's PBS either with or without calcium and magnesium and ACK lysis buffer were from Gibco Laboratories (Grand Island, NY). Lymphocyte separation medium (LSM) was from Organon Teknika Corp. (Durham, NC). Colloidal gold probes (5 and 10 nm) conjugated with either goat anti–rabbit IgG (GAR 5 or GAR 10) or goat anti–mouse IgG (GAM 5 or GAM 10) were purchased from Amersham Corp. (Arlington Heights, IL). Sea Plaque low-gelling temperature agarose was obtained from FMC BioProducts (Rockland, ME). Glutaraldehyde and osmium tetroxide were from Electron Microscopy Sciences (Fort Washington, PA), paraformaldehyde was from Fisher Scientific (Fair Lawn, NJ) and A23187 and DMSO and other chemicals were of reagent grade and were obtained from Sigma Chemical Co. Recombinant 5-LO was provided by Dr. Denis Riendeau, Merck Frosst, Pointe Claire-Dorval, Canada.

Isolation, Fixation, and Freezing of Human Peripheral Blood Monocytes and PMN. Leukocytes were isolated from venous blood of normal human volunteers by dextran sedimentation followed by centrifugation through LSM and hypotonic lysis to remove residual erythrocytes (22). The resulting PMN were then washed in PBS without calcium and magnesium. Monocytes, which collect in the boundary layer between LSM and the overlaying PBS, were also harvested. Final cell pellets were resuspended in Dulbecco's PBS containing calcium and magnesium at a cell concentration of 2–4 $\times 10^6$ cells per ml. In certain experiments cells were activated by incubation in 5 μg/ml of the calcium ionophore A23187 for 5 min at 37°C before subsequent processing.

After isolation, and in some experiments, activation, $\sim 5 \times 10^6$ cells suspended in a total volume of 2.5 ml of ice cold PBS were mixed with an equal volume of a 2× fixative prepared to yield final concentrations of 0.05% glutaraldehyde, 3.5% paraformaldehyde, and 0.1 M sucrose in PBS, pH 7.3. This mixture was irradiated in a microwave oven for a period (typically 20–22 s) sufficient to raise the temperature of the suspension to 45°C (28). After irradiation the glutaraldehyde was immediately quenched by the addition of 10 ml of paraformaldehyde-lysine-periodate (PLP) fixative (29). The quenched cells were washed twice in additional PLP and fixation was continued in PLP overnight at 4°C. The following morning, the fixed cells were pelleted by centrifugation at 1000 *g* for 5 min and resuspended in 1 ml of PBS containing 0.1 M sucrose. These cells were mixed with an equal volume of 2% low-gelling temperature agarose and pelleted in 400-μl pointed microfuge tubes in a prewarmed swinging bucket microfuge rotor such that the cells formed a tight pellet before the agar hardened. Typically 3–6 $\times 10^6$ cells were divided into 10 pellets at this point. The agar embedded cells were cryoprotected by infiltration for 90 min with 2.3 M sucrose in phosphate buffer, pH 7.2 containing 50% polyvinylpyrrolidone (30) after which they were mounted on aluminum nails and frozen by injection into liquid propane at −185°C in a freezing apparatus (Model KF-80; Reichert Scientific Instruments, Buffalo, NY). Frozen samples were then stored under liquid nitrogen until use. In certain experiments, cells were fixed for 30 min in 0.01% glutaraldehyde/3.0% paraformaldehyde in PBS or overnight in PLP fixative. However the morphology of the final sections obtained with these fixations was inferior to that obtained with the microwave/glutaraldehyde/PLP procedure. Each experimental observation was verified in at least three independent batches of cells isolated from different donors.

SDS-PAGE and Immunoblot Analysis. Human leukocytes were isolated from buffy coat concentrates (Red Cross, Montreal, Canada), sonicated, and then fractionated by differential centrifugation as previously described (22). Proteins from the 100,000 *g* pellet (for FLAP immunoblots, 1.2 μg protein/lane), from 30–60% ammonium sulfate precipitated 10,000-*g* supernatant fractions (22) (for rabbit anti–5-LO blots, 12 μg protein/lane) or purified human 5-LO (for mouse anti–5-LO blots, 0.5 μg/lane) were separated by SDS-PAGE. Separated proteins were electrophoretically transferred to nitrocellulose overnight at 100 mA using a Novex immunoblot apparatus (Novel Experimental Technology, San Diego, CA). Immunoblot analysis was then carried out as previously described (16) using: (*a*) Protein A purified IgGs at a dilution equivalent to a 1/200 dilution of unpurified antisera for rabbit anti-FLAP IgG and rabbit anti–5-LO IgG and their corresponding preimmune IgGs; and (*b*)

a 1/250 dilution of mouse anti-5-LO antisera and its preimmune serum. ^{125}I-Protein A (NEN-Dupont, Mississauga, Ont., Canada) was used to detect bound antibodies. In the case of mouse antisera, the transfers were also incubated with a 1/500 dilution of affinity purified goat anti-mouse IgG (Cappel, Scarborough, Ont., Canada) before incubation with ^{125}I-Protein A.

For immunoblot analysis of subcellular fractions, the proteins from each fraction were first concentrated by adding 55 μl ice cold 100% TCA to a 500 μl suspension of material. After 30 min at 4°C the precipitated proteins were harvested by centrifugation for 10 min at 13,000 *g* at 4°C. The supernatant was discarded and the pellet resuspended in SDS-PAGE sample buffer, adjusted to neutrality with NaOH and boiled before SDS-PAGE. After electrophoresis, the proteins were transferred to nitrocellulose, which was then cut into strips and incubated with a 1:500 dilution of FLAP antisera or 1:300 dilution of PMN13F6 ascites fluid and processed as above. The 18-kD bands, corresponding to FLAP, of the resultant autoradiographs were scanned using a 2202 Ultrascan Laser densitometer (LKB Instruments, Inc., Gaithersburg, MD). For each sample, duplicate 5 and 10 μl aliquots of the SDS sample were loaded on to the gels and in all cases the lanes containing 10 μl had approximately double the density of the lanes containing 5 μl.

Immunogold Labeling of Cryosections. Ultrathin cryosectioning was carried out at −105°C following the techniques of Tokuyasu (31) on a Reichert Ultracut S ultramicrotome equipped with an FCS cryoattachment. Sections were transferred to hexagonal nickel grids (200-mesh) that had been coated with formvar and glow cleaned just before use. All incubations and washing steps were carried out by floating the grids on droplets of solution dispensed through 0.2 μm syringe filters. After two 10-min incubations with 1% BSA in PBS containing 0.02% sodium azide (BSA/PBS) to block nonspecific binding, the sections were incubated overnight at 4°C with primary antibody solutions at 5 μg/ml in BSA/PBS. To demonstrate specificity, in certain experiments the primary antibodies were preincubated with either recombinant 5-LO or the peptide corresponding to amino acids 41–52 of FLAP at 10 μg/ml for 30 min followed by centrifugation at 10,000 *g* for 10 min before incubating the resultant supernatant fraction with sections. The following morning, the sections were washed extensively with BSA/PBS and then incubated with 5- or 10-nm immunogold probes for 60 min at room temperature. Anti-rabbit probes were diluted 1:50 and anti-mouse probes were diluted 1:25 in BSA/PBS. After the gold incubation the grids were again washed extensively in BSA/PBS, fixed in 2% glutaraldehyde in PBS for 10 min, postfixed in 2% osmium in H_2O for 20 min, stained with 2% uranyl acetate in H_2O for 30 min, and finally absorption stained with 0.002% lead citrate in 2% polyvinylalcohol as described by Tokuyasu (30). Grids were examined and micrographs taken in an electron microscope (100CX; JEOL USA, Electron Optics Division, Peabody, MA) at 80 kV at an initial magnification of 19,000.

Isolation of Nuclei and FLAP Inhibitor Binding. A suspension of leukocytes containing 2 × 10^7 cells was centrifuged at 500 *g* for 10 min to form a loose pellet which was resuspended in 1 ml of NP40 lysis buffer (0.1% NP40, 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA, and 1 mM PMSF). This suspension was vortexed for 15 s and then centrifuged at 300 *g* for 10 min at room temperature. The resultant supernatant fraction (the nonnuclear fraction) was divided into two 500-μl aliquots, one of which was stored at −70°C for FLAP binding analysis while the other was precipitated with TCA and processed for SDS-PAGE and immunoblot analysis. The NP-40 lysis pellet fraction (nuclei) was resuspended in 90 μl of DNase 1 buffer (20 mM Hepes, pH 7.5, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM EDTA, and 1 mM PMSF) and 10 μl DNase 1 (1 mg/ml) was added and incubated for 30 min at 37°C. The DNase treated nuclei fraction was divided into two equal 50-μl samples. These samples were diluted to 1 ml by the addition of 950 μl Hepes lysis buffer and sonicated as above. A 10-μl aliquot of the this material was observed by light microscopy to verify that >95% of the nuclei were lysed. One sample was then precipitated with TCA and processed for SDS-PAGE and immunoblot analysis. The second sample was centrifuged at 100,000 *g* for 60 min at 4°C in a TL-100 Ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA). The supernatant containing nucleoproteins was precipitated with TCA and processed for SDS-PAGE. The pellet was resuspended in 1 ml Hepes lysis buffer, sonicated, precipitated with TCA, and processed for SDS-PAGE.

The FLAP inhibitor binding assay was modified from the method of Charleson (32). This assay is based on the observation that MK-886, an indole leukotriene biosynthesis inhibitor, binds with high affinity and specificity to FLAP (15). Aliquots from leukocyte preparations were resuspended in assay buffer (100 mM Tris-HCl, pH 7.5, 140 mM NaCl, 2 mM EDTA, 0.5 mM dithiothreitol, 5% glycerol, 0.05% Tween 20). Duplicate assays at two protein concentrations for each sample were performed in Beckman 96–deep-well polypropylene microtiter plates. Binding was initiated by the addition of leukocyte samples (1–15 μg protein) to wells containing 100 μl ^{125}I-L-691-831 (a radiolabeled analog of MK-886), 300–700 pM final concentration, diluted in assay (buffer/methanol, 97:3; vol/vol) and either 2 μl Me_2SO or MK-886 in Me_2SO (10^{-5} M final concentration), plus the required volume of assay buffer to bring the final volume to 200 μl. The plate was incubated with shaking for 20 min at room temperature. The samples were then filtered and washed using a Harvester 96 (Tomtec, Orange, CT) onto a LKB printed filtermat B, prewetted with wash buffer (100 mM Tris-HCl, pH 7.5, 0.05% Tween 20). Bound ^{125}I was determined in a LKB 1272 Clinigamma Quatro counter. Specific binding was defined as the difference between binding in the absence and the presence of 10^{-5} M MK-886.

To quantitate total FLAP protein and inhibitor binding present in whole cells, a second suspension of leukocytes containing 2 × 10^7 cells was centrifuged at 500 *g* for 10 min. The pelleted cells were then resuspended in 1 ml of Hepes lysis buffer (5 mM Hepes, pH 7.4, 1 mM EDTA, and 1 mM PMSF). This suspension was sonicated on ice by three 20 s bursts using a Cole-Palmer 4710 ultrasonic homogenizer set at 70% output (Chicago, IL). Cell lysis was checked visually and if lysis was not >95% the sample was resonicated. The complete sonicated suspension was divided into two 500-μl aliquots, stored, and processed for SDS-PAGE and FLAP inhibitor binding as described above.

Results

Characterization of Antibody Probes. The specificity of the anti-FLAP and anti-5-LO probes used in the immunolocalization experiments was verified by immunoblot analysis. Crude protein fractions from human leukocytes were separated by SDS-PAGE, transferred to nitrocellulose and analyzed. Fig. 1 demonstrates that anti-FLAP rabbit IgG recognizes a single 18-kD protein that has been previously identified as FLAP (15). Fig. 1 also shows that rabbit anti-5-LO IgG and mouse polyclonal 5-LO antiserum bind most predominantly to a single protein of 78 kD that has been identified as 5-LO (22). The rabbit antiserum also immunoprecipitated a ^{35}S-labeled 78-kD protein from DMSO differentiated HL-60 cells prela-

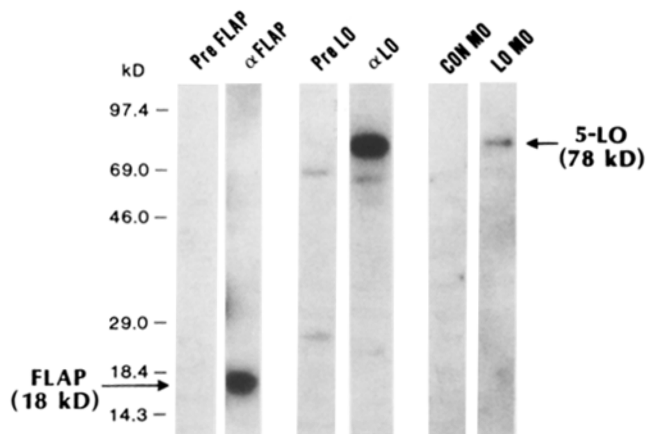


Figure 1. Immunoblot characterization of 5-LO and FLAP antibodies. Human PMN proteins on nitrocellulose strips were incubated with: lane *1*, preimmune IgG (from the anti-FLAP rabbit); lane *2*, anti-FLAP IgG; lane *3*, preimmune IgG (from the anti-5-LO rabbit); lane *4*, anti-5-LO IgG; lane *5*, control mouse serum; and lane *6*, mouse anti-5-LO serum, as described in Materials and Methods. Note that anti-5-LO IgG reacts with a single band of 78 kD, that anti-FLAP IgG detects a single protein of 18 kD, and that the preimmune IgGs only react weekly with nonspecific bands. Also note that mouse anti-5-LO serum detects a band at the migration position of authentic 5-LO, whereas control mouse serum does not label this band. Molecular mass markers are shown to the left and the migration positions of 5-LO (78 kD) and FLAP (18 kD) are indicated.

beled with [^{35}S]methionine (24). The precipitation of this labeled protein could be completely eliminated by preincubating the anti-5-LO with excess unlabeled purified 5-LO (Ethier, D., and J. Evans, unpublished data).

FLAP Is in the Nuclear Envelope of Resting Cells. To visualize the intracellular distribution of FLAP and 5-LO in resting cells, freshly isolated peripheral blood leukocytes were fixed and frozen at −185°C. Ultrathin cryosections of the frozen cells were incubated with either anti-FLAP or anti-5-LO IgG followed by colloidal gold conjugated secondary probes. Electronmicroscopic observation of the sections incubated with anti-FLAP antibodies revealed that FLAP was strikingly concentrated in the lumen of the nuclear envelope of both neutrophils (Fig. 2 *A*) and monocytes (Fig. 2 *B*). In rare instances anti-FLAP is also observed to label the endoplasmic reticulum of some monocytes (Fig. 2 *B*). This endoplasmic reticulum labeling is most likely a reflection of anti-FLAP binding to newly synthesized, possibly inactive, FLAP protein, which would be expected to be translated on rough endoplasmic reticulum. Since cells displaying this labeling pattern are only rarely observed, it is likely that they represent a subpopulation of cells that had been partially activated in the donor before isolation.

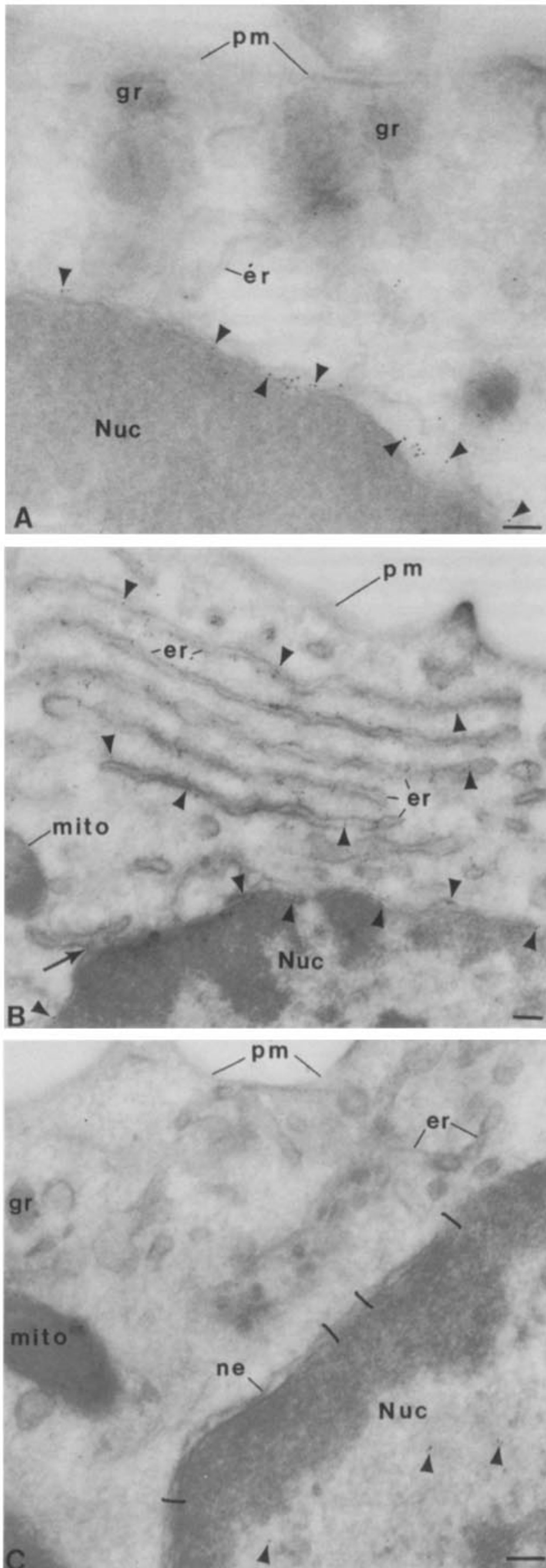
Immunogold labeling was not observed along the plasma membrane or associated with any other membrane structures.

Table 1. *Immunoblot Analysis* and Quantitation of FLAP Inhibitor Binding‡ on Isolated Leukocyte Fractions*

	Whole cell lysates	Nonnuclear (300-*g* supernatant)	Nuclear (300-*g* pellet)	Nucleoplasmic (100,000-*g* supernatant)	Nuclear membrane (100,000-*g* pellet)
Immunoblot analysis of FLAP	100%	2 ± 3%	141 ± 57%	0 ± 0%	83 ± 39%
Immunoblot analysis of PMN13F6	100%	109 ± 56%	3 ± 5%	0 ± 0%	0 ± 0%
FLAP inhibitor bound (pmol/mg protein)	1.0 ± 0.4	0.2 ± 0.1	3.3 ± 1.0	0.1 ± 0.1	4.1 ± 0.9
Total FLAP inhibitor bound (pmol/10^7 cells)	1.25	0.2	1.0	0.01	0.8

* Immunoblot analysis of FLAP and PMN13F6 antigen from whole cell lysates and subcellular fractions of resting human leukocytes was carried out as described in Materials and Methods. For each determination, identical aliquots containing 5 × 10^5 cells were either lysed and loaded directly on to SDS-PAGE gels or fractionated as described in Materials and Methods before immunoblot analysis. The average density of duplicate bands for each fraction was determined and normalized to the density of the whole cell lysate which was defined as yielding 100%. Percentages were calculated for three separate experiments and are shown ± SD.

‡ FLAP inhibitor binding to human leukocyte lysate and subcellular fractions were performed as described in Materials and Methods. Three separate experiments were performed in duplicate and the values were averaged ± SD.



In our sections neutrophils were identified by their very polymorphic nuclei and the presence of large numbers of characteristic granules, whereas monocytes had rounder nuclei, contained fewer granules, and had much more extensive endoplasmic reticulum. Sections incubated with anti-5-LO IgG were devoid of any detectable gold label along either the nuclear envelope, the endoplasmic reticulum, or the plasma membrane. In addition, sections of resting leukocytes incubated with either anti-FLAP or anti-5-LO IgG did not display any label associated with the Golgi complex, mitochondria, membrane bound granules, or within the cytoplasmic ground substance (Fig. 2 *C*). However, there were occasionally a few gold particles observed over the euchromatin region within the nucleus of sections incubated with anti-5-LO IgG (Fig. 2 *C*). Resting (no treatment before fixation) and sham-treated (DMSO vehicle only) leukocytes gave identical labeling patterns.

To confirm the immuno-EM observation (Fig. 2, *A* and *B*) that FLAP was present in the nuclear envelope, we prepared nuclear and cytoplasmic fractions from isolated resting human leukocytes. The proportion of FLAP determined by immunoblot analysis and the distribution of FLAP ligand binding activity between fractions was then determined. The immunoblot results, summarized in Table 1, show that FLAP protein resides predominantly in the nuclear fraction, which was isolated as a 300-*g* pellet of NP-40 lysed cells. When the amount of FLAP detected in whole cell lysates is defined as 100%, the nuclear fraction contains 141% of this amount, whereas the 300-*g* supernatant fraction only contains 2%. If the nuclear fraction was further divided into nuclear membrane and nucleoplasm fractions by sonication of the 300-*g* nuclear pellet followed by a 100,000-*g* centrifugation step, 83% of the amount present in the whole cell lysate was recovered in the 100,000-*g* pellet whereas 0% remained in the supernatant fraction. As a fractionation control, a similar analysis was carried out utilizing PMN13F6, an antibody that recognizes a neutrophil plasma membrane protein (26, 27). This control (Table 1) showed that <3% of the PMN13F6 antigen was found in the nuclear fraction, indicating that the separation of nuclei and plasma membrane was clean. The apparent increase in the absolute amount of FLAP in the nuclear fraction relative to the whole cell fraction may be an artifact of the quantitation procedures since the whole cell pellets were more difficult to disperse in sample buffer, or may be the result of increased antigenicity of FLAP in the

Figure 2. Immunogold labeling of ultrathin frozen sections of resting leukocytes incubated with anti-FLAP IgG (*A*, *B*) or anti-5-LO IgG (*C*). Note that FLAP immunogold label (*arrowheads*) is concentrated within the lumen of the nuclear envelope in neutrophils (*A*) and along the nuclear envelope and endoplasmic reticulum of monocytes (*B*). In *B* it is particularly clear that the nuclear envelope is contiguous with the endoplasmic reticulum (*arrow*). Ultrathin frozen sections of resting leukocytes incubated with anti-5-LO display only a small number of gold particles over the euchromatin region of the nucleus (*arrowheads*, *C*). The lengths of nuclear envelope delineated by brackets are representative of well-preserved regions in which both the inner and outer leaflet of the nuclear envelope can be clearly observed. These and similar lengths from additional micrographs were analyzed to provide the data presented in Tables 1-3. Nuc, nucleus, gr, granule, bar, 100 nm.

nuclear fraction. It is also possible that the proteins in the whole cell fraction may have been more susceptible to proteolysis than the NP-40 lysed fractions.

The results of FLAP inhibitor binding experiments (Table 1) also demonstrate that ~64% (0.8/1.25) of the FLAP inhibitor binding activity fractionates with the nuclei and the vast majority of this nuclear material was associated with the nuclear membrane (4.1 vs. 0.1 pmol/mg protein). Thus, results of both immunoblot quantitation of FLAP protein and direct binding of iodinated FLAP inhibitor confirm the immuno-EM observation that FLAP is concentrated in the nuclear envelope of resting leukocytes.

FLAP and 5-LO Are in the Nuclear Envelope of Activated Cells. To determine the effect of activation on the intracellular distribution of FLAP and 5-LO, freshly isolated leukocytes were incubated with the calcium ionophore A23187 before fixation, freezing, cryosectioning, and immunolabeling. As in resting cells, sections incubated with anti-FLAP antibodies displayed immunogold labeling within the lumen of the nuclear envelope (Fig. 3, *A* and *B*). Moreover, in marked contrast to resting cells, ionophore-activated cells incubated with anti–5-LO IgG displayed immunolabeling within the lumen of the nuclear envelope (Fig. 3, *C* and *D*). This pattern was very similar to that obtained with anti-FLAP antibodies. Both antibodies also occasionally labeled the endoplasmic reticulum. However, the density of gold particles observed here was much less concentrated than that found in the lumen of the nuclear envelope (data not shown). Neither antibody yielded any significant labeling of the plasma membrane, nucleus (exclusive of the nuclear envelope), Golgi complex, mitochondria, membrane bound granules, or cytoplasmic ground substance. Similar patterns of 5-LO and FLAP distribution were observed in both neutrophils and monocytes. In addition, with either probe there was very little background labeling observed outside of the cells.

In an effort to quantitate the preceding observations, the number of gold particles observed along measured lengths of the nuclear envelope were counted (Table 2). These results show that in cells labeled with anti-FLAP IgG there were 12 and 17 particles/μm, in resting and activated cells, respectively. In cells labeled with anti–5-LO there were 1 and 11 particles/μm, in resting and activated cells, respectively. This analysis underscored the dramatic increase in 5-LO labeling that calcium ionophore activation produced in these cells. It also suggested that activation did not have any significant effect on the distribution or density of FLAP labeling. It should also be noted that for the purposes of this analysis only regions of the nuclear envelope that were very well preserved, in which both the inner and outer lipid bilayers were evident and in which the lumen was not dilated were considered. Examples of typical areas included for this analysis are delineated by brackets in Figs. 2 and 3.

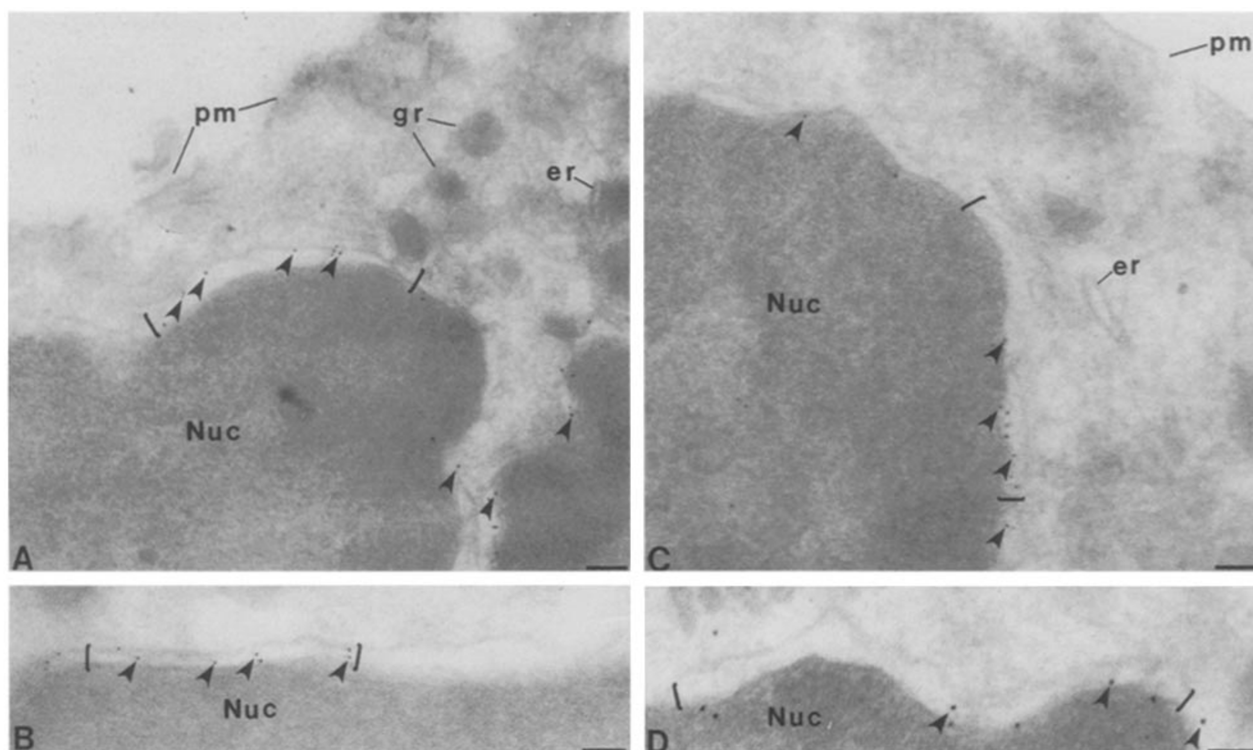


Figure 3. Immunogold labeling of ultrathin frozen sections of ionophore activated neutrophils incubated with anti-FLAP IgG (*A*, *B*) or anti–5-LO IgG (*C*, *D*). Both FLAP and 5-LO antibodies label the lumen of the nuclear envelope (*arrowheads*). Gold particles are not observed associated with the plasma membrane (pm), or membrane bound cytoplasmic granules (gr). Neither IgG appears to label the nuclear matrix or the cytoplasmic ground substance. Lengths of nuclear envelope delineated by brackets are typical of those analyzed to provide the data presented in Table 2. Nuc, nucleus, er, endoplasmic reticulum, bar, 100 nm.

Table 2. *Quantitation of Anti-FLAP and Anti-5-LO labeling of the Nuclear Membrane in Activated and Sham-treated Leukocytes**

	Sham-treated	Ionophore-activated
anti-FLAP	12.3 ± 5.9 (8)	16.8 ± 7.8 (9)
anti-5-LO	0.9 ± 0.26 (21)	11.1 ± 3.5 (8)

* Data presented as the mean number of particles/μm ± SD (number of cells counted).

The distribution of immunogold particles within the nuclear envelope was also quantitated in a similar manner (Table 3). In this analysis gold particles were counted as being associated with either the inner or the outer nuclear membrane if they were touching or within one particle diameter of that membrane. Other particles, not within one diameter of either membrane but still within the lumen, were considered to be luminal. Particles that appeared to be outside of either membrane were not included in this analysis. The results show that both FLAP and 5-LO were relatively more concentrated on the inner nuclear membrane than on either the outer membrane or within the lumen. The distribution of 5-LO was heavily skewed toward the inner nuclear membrane; 50% of the bound gold particles were observed associated with the inner membrane, <40% appeared to be in the lumen, and ~10% were associated with the outer membrane. FLAP staining was also more often associated with the inner membrane; >40% of the immuno-FLAP gold particles were localized on the inner membrane, ~25% of the label appeared to be in the lumen, and <30% was associated with the outer nuclear membrane. In this analysis, it was implicitly assumed that both 5-LO and FLAP were associated with a membrane after activation as was suggested by subcellular fractionation experiments (14, 33). Thus, immunogold particles that were counted as being in the lumen were probably physically associated with either the inner or outer nuclear membranes, but could not be unambiguously assigned as being associated with either. Therefore, it was likely that an even higher proportion of both 5-LO and FLAP were associated with the inner nuclear membrane than was reflected by these data.

In a related experiment, carried out to determine if FLAP or 5-LO was expressed at the plasma membrane, freshly isolated, ionophore activated cells were incubated with FLAP or 5-LO antibodies before fixation. This incubation was carried out at 4°C in order to eliminate the possibility of endocytosis of surface bound antibody. After fixation and processing, sections were incubated with immunogold probes. An additional objective of this approach was to determine if fixation inhibited immunolabeling of putative cell-surface FLAP or 5-LO epitopes. The results of this experiment were that no surface bound gold label could be detected on cells incubated with these antibodies before fixation. However, if the same sections were incubated with anti-FLAP or anti-5-LO antibodies after sectioning, the nuclear envelope was clearly labeled (data not shown).

5-LO and FLAP Can Be Colocalized in the Nuclear Envelope. Double label experiments were performed to verify that both 5-LO and FLAP were present within the same cellular compartment at the same time. In these experiments cryosections of A23187 activated leukocytes were first incubated with a mixture of mouse anti-5-LO and rabbit anti-FLAP antibodies, followed by a mixture of the species specific immunoprobes GAM 10 and GAR 5. It is important to note that these two gold probes are sufficiently different in size to be readily distinguished in the electronmicroscope. The resulting doubly stained micrographs (Fig. 4) showed that both 5-LO and FLAP epitopes were present within the nuclear envelope of the same cells. Both probes were observed clustered together in close proximity (Fig. 4, *boxed area*) but were also found randomly scattered within these compartments. This demonstrates that FLAP and 5-LO can be simultaneously observed within the same compartments at the same time and are often in close proximity to one another. Identical results were obtained if GAM 5 and GAR 10 were used instead of GAM 10 and GAR 5. As a further control for the double label protocol, cryosections were incubated with only one of the primary antibodies followed by the mixture of both gold probes. In these cases only the appropriately sized gold probes bound to the sections, demonstrating that the localization of the secondary reagents was specific for the the primary reagents (data not shown).

Demonstration of Specificity of Antibody Labeling. A number of control experiments were carried out to demonstrate that the labeling patterns described above were specific. In many

Table 3. *Distribution of Anti-FLAP and Anti-5-LO Label Across Nuclear Envelope of Activated Leukocytes**

	Inner membrane	Lumen	Outer membrane
Anti-FLAP	41.8 ± 18.0%	24.4 ± 15.8%	27.4 ± 10.0%
Anti-5-LO	49.8 ± 11.3%‡	39.1 ± 14.8%§	11.0 ± 14.7%‡§

* Percentage of gold particles within one particle diameter of the luminal face of the inner or outer nuclear membrane. All particles not within one diameter of either membrane were counted as being in the lumen. At least eight different cells were counted for each condition.
‡ Significantly different from each other, $p < .005$.
§ Significantly different from each other, $p < .025$.

of our immunolocalization experiments a group of sections were incubated with a monoclonal IgG specific for CD18 (clone H52) rather than for FLAP or 5-LO epitopes. The results with this antibody (Fig. 5) showed that CD18 was localized in granules and on the plasma membrane in neutrophils and monocytes, but was absent from the nuclear envelope and the endoplasmic reticulum. Similar CD18 patterns have been previously reported (34–36). Since this pattern was obtained using the same immunogold reagent as was used in the double label experiment (GAM 5), it indicates that the labeling of the nuclear envelope and endoplasmic reticulum with anti-FLAP and anti–5-LO could not be the result of nonspecific interactions of either the primary IgG or the gold probe with an unidentified component within these compartments.

Cryosections of both activated and resting leukocytes were also incubated with preimmune IgG and the appropriate colloidal gold probes. In every case, no significant labeling of the nuclear membrane, the plasma membrane or any other cellular structure or compartment was observed when sections were labeled with preimmune IgG (data not shown).

Another control experiment that demonstrated the specificity of the labeling patterns observed with anti-FLAP and anti–5-LO was to preincubate the primary antibodies with the appropriate free antigen before incubation with cryosections. The percent inhibition was then calculated by dividing the number of gold particles/μm nuclear membrane counted on sections incubated with antibodies preincubated with free antigen by that obtained from sections incubated with untreated antibody. When anti-FLAP was preincubated with 5 μg/ml of the appropriate FLAP peptide, labeling was reduced by >90%. When anti-5-LO was preincubated with 5 μg/ml recombinant 5-LO, ~75% of the labeling was eliminated (Table 4).

An additional control for antibody specificity was obtained by observing cells present as contaminants in leukocyte preparations that are not known to contain significant amounts of either FLAP or 5-LO. T and B lymphocytes in particular have been reported to contain very little, if any, of either of these proteins (7). Lymphocytes are readily distinguished in our cryosections by their large round nucleus and sparse cytoplasm. Cells of this morphology in our preparations were completely devoid of any detectable FLAP or 5-LO immunolabeling. In a related, separate experiment, we were also unable to immunolabel a human cytotoxic T cell line (designated Q31) with either 5-LO or FLAP IgGs (data not shown).

A final control for antibody specificity was provided by the effect of A23187 on the localization of 5-LO. Our initial

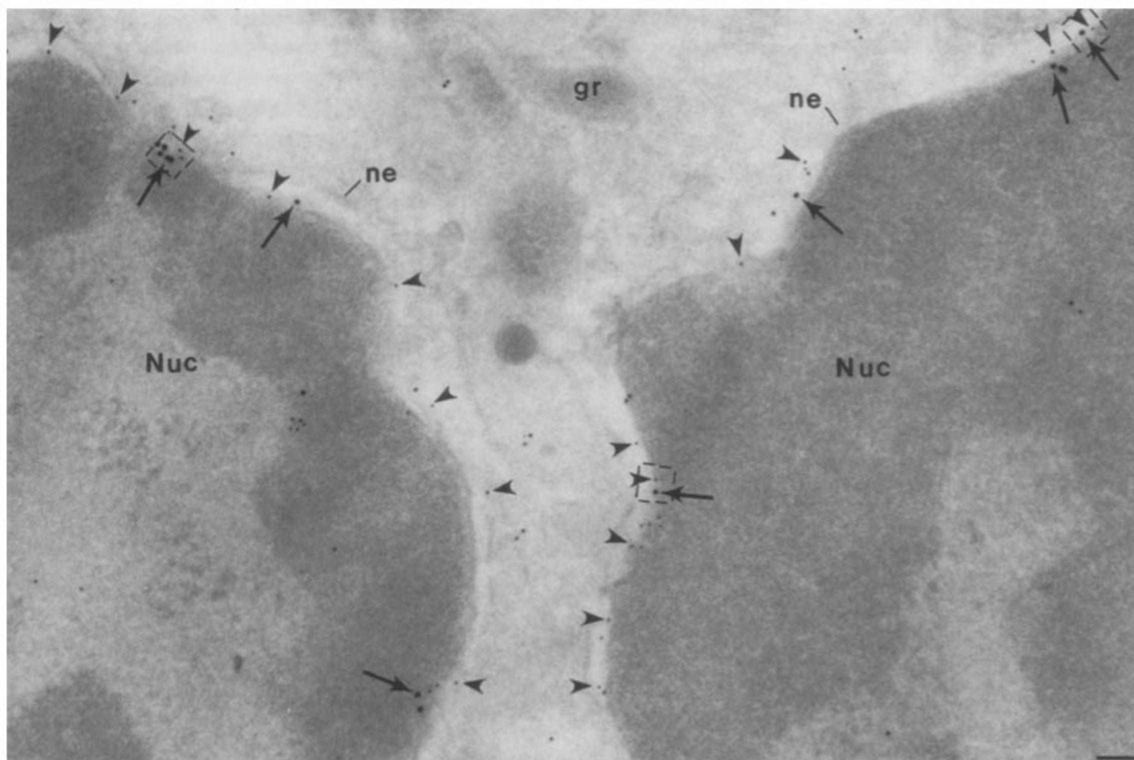


Figure 4. Double immunogold labeling of 5-LO (10-nm gold, arrows) and FLAP (5-nm gold, arrowheads) on ionophore-activated monocyte. Note that both labels are observed within the lumen of the nuclear envelope. Also notice that in some regions the labels are randomly distributed along this compartment but are occasionally clustered together (boxed area). Both labels are also observed associated with endoplasmic reticulum cisternae (er). Neither label is observed over the cytoplasmic ground substance. These specimens were prepared by incubating sections of activated leukocytes with a mixture of mouse anti–5-LO and rabbit anti-FLAP, washing, and then incubating with a mixture of GAM 10 and GAR 5. Nuc, nucleus, gr, granule, bar, 100 nm.

Table 4. *Inhibition of Antibody Labeling by Preincubation with Excess Free Antigen**

	Antibody only	Antibody plus free antigen	Percent inhibition
Anti-FLAP	12.9 ± 4.7 (6)	1.1 ± 0.4 (6)	91.4%
Anti–5-LO	13.3 ± 3.4 (6)	3.5 ± 0.5 (6)	73.3%

* Data presented as the mean number of particles/μm ± SD (number of cells counted).

experiments demonstrated that in resting cells it was virtually impossible to immunolabel this enzyme, whereas after 5 min of ionophore activation, 5-LO could be readily visualized within the lumen of the nuclear envelope and endoplasmic reticulum. Since the same reagents were used in either case, the change in labeling would most likely have been the result of a specific event that occurred as a result of activation. This event was most probably the previously well described translocation of 5-LO from a 100,000-*g* supernatant form in resting cells to a membrane-associated form in activated cells (4, 33).

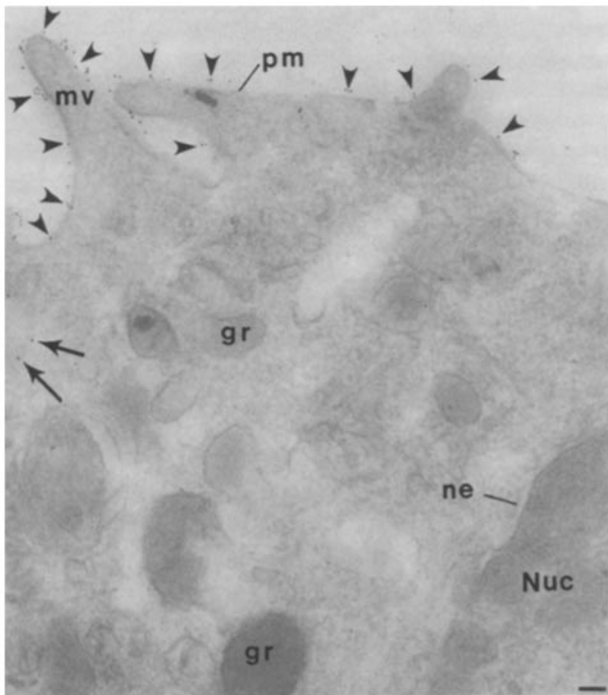


Figure 5. Immunolocalization of CD18 on an activated neutrophil. The gold immunolabel is observed along the plasma membrane (pm, *arrowheads*) where it is more often observed on microvilli (mv) but is also present in areas between microvilli. Immunoreactive CD18 is also occasionally observed within small membrane bound cytoplasmic granules (*arrows*). Label is not associated with the nuclear envelope (ne) or the endoplasmic reticulum (er), nor are the gold particles found over the cytoplasmic ground substance or within the nuclear matrix. Background label outside of the cell is very rarely observed. Nuc, nucleus, bar, 100 nm.

Discussion

Our immunolocalization data demonstrated that in ionophore-activated monocytes and PMN's, both FLAP and 5-LO were localized in the lumen of the nuclear envelope. In addition, except for very weak labeling of the endoplasmic reticulum that may represent newly synthesized protein in the biosynthetic pathway, neither protein could be detected in any other cell compartment or along the plasma membrane. In resting cells, the FLAP distribution was identical to that observed in activated cells. In contrast, sections of resting or sham-treated leukocytes incubated with anti–5-LO IgG did not display any detectable gold label along the nuclear envelope, endoplasmic reticulum, or plasma membrane, or within the Golgi complex, other membrane bound organelles, or over the cytoplasmic ground substance. However, there were occasionally a few gold particles observed over the euchromatin region within the nucleus. The subcellular fractionation of resting leukocytes into nuclear and nonnuclear fractions also demonstrated that the vast majority of the total cellular FLAP protein was associated with the nuclear membrane. Our observation that 5-LO translocates to the permanent site of FLAP residence after activation, combined with the literature reporting that FLAP is required for in vivo formation of leukotrienes (15–17), demonstrate that the nuclear envelope comprises the intracellular site where 5-LO and FLAP act on arachidonic acid to form 5-HPETE and LTA_4. These data further suggest that the morphological correlate of the biochemically-defined translocation event (14, 33) is the movement of 5-LO from a nonsedimentable location to the nuclear envelope.

This conclusion is supported by biochemical evidence that makes it clear that in several cell types the nuclear envelope itself is the principle source of arachidonate for eicosanoid synthesis. Eicosanoids are the family of molecules derived from arachidonic acid and include the 5-LO products i.e., leukotrienes, as well as products of cyclooxygenase, i.e., prostaglandins. Electronmicroscopic autoradiography of a mouse fibrosarcoma cell line has shown that [^{3}H]arachidonic acid is most rapidly incorporated into the nuclear membrane (37) and further that recently incorporated arachidonate is most efficiently utilized for eicosanoid synthesis. Similarly, when [^{14}C]arachidonate was incorporated into mouse fibroblasts, cell fractionation experiments demonstrated that this label was incorporated most rapidly into a nuclear fraction and

that this fraction provided the bulk of the material that could be subsequently incorporated into eicosanoids in response to bradykinin stimulation (38). Thus, it would seem likely that the enzymes required for the initial step of eicosanoid synthesis would be localized along the nuclear envelope. Indeed, prostaglandin endoperoxidase synthase 1 and 2 has been localized in mouse 3T3 fibroblasts by indirect immunofluorescence to the nuclear envelope and ectoplasmic reticulum (39).

The observation that the nuclear envelope is a key site of eicosanoid metabolism is not widely understood. Indeed, in many preparative procedures, the implicit assumption is that the nuclear fraction can be discarded in the process of preparing cell-free homogenates. In particular, in the field of leukotriene research, there are several articles (33, 40, 41) on the biochemistry of 5-LO metabolism in which cells were first homogenized and the 10,000-*g* pelletable material discarded before analysis of subsequent subcellular fractions. However, our results indicate that by discarding the low speed pellet before analysis of activated leukocytes, a significant portion of the total cellular 5-LO must also have been discarded, thus adversely affecting the ability to understand the biology of the processes under study.

The apparent difficulty in visualizing the intracellular distribution of 5-LO in resting cells may be an indication of the lack of an association of this protein with any membrane, cytoskeletal, or other elements that are retained in the final ultrathin frozen sections. Since the sections were cut before incubation with antibodies, this result suggests that in resting cells 5-LO was neither fixed in place itself nor was it associated with a fixed structure, but rather the majority of this protein was washed out of the sections during the blocking, antibody incubation, and washing steps. This should not be surprising in that sequence analysis of 5-LO provides no indication of either hydrophobic domains that may associate directly with a lipid bilayer, nor of leader sequences that would mediate translation on rough endoplasmic reticulum (42). In addition, 5-LO has shown no evidence of isoprenylation or phosphorylation, which could also potentially mediate membrane or cytoskeletal associations (our unpublished observations). Alternately, it may not have been possible to visualize 5-LO in resting cells because the particular epitopes that bind the anti–5-LO antibody used in these experiments were masked, or because fixation cross-linked these epitopes (in resting cells only) in such a way that the antibodies could not bind. In any event, activation leads to a fundamental change in the ability of anti–5-LO antibodies to label ultrathin frozen sections and this property correlates very well with previous reports describing the "translocation" of 5-LO from a cytosolic to a membrane fraction (14, 33).

Our immunolocalization experiments utilized a novel combination of fixation techniques that has not previously been described. We found that the combination of a very brief fixation in 0.05% glutaraldehyde/3.5% paraformaldehyde during which the cells were heated to 45°C by microwave irradiation (originally described by Dvorak et al. [28]), followed by several washes and an overnight incubation at 4°C in PLP fixative (29) yielded a superior combination of retained antigenicity and well-preserved ultrastructure. In particular this scheme preserved the content of many of the PMN granules, which are often lost with other fixation techniques. We compared this procedure with other protocols previously utilized to preserve PMN for ultrastructural immunolocalization experiments. Alternate procedures included 0.05–1.0% glutaraldehyde in phosphate buffer for 30 min at 4°C (34) and combinations of glutaraldehyde and paraformaldehyde incubated at 4°C or 23°C for 30 min (36). These procedures were less effective than the final glutaraldehyde/paraformaldehyde microwave/PLP protocol used here.

Immunogold labeling of ultrathin frozen sections has a significant advantage over other postfixation immunolabeling techniques. When labeling ultrathin frozen sections, accessibility to intracellular compartments is obtained by virtue of the fact that membrane bound compartments are cut open. Thus, antibody probes have ready access to epitopes on either side of any membrane bound compartment. However, since the sections are incubated with the primary antibodies after fixation, there remains the possibility that fixation of critical epitopes may interfere with antibody recognition. Experiments in which cells were incubated with anti-FLAP or anti–5-LO antibodies before fixation were carried out to address this possibility. The results of these experiments showed that surface bound gold label could not be detected on sections of cells incubated with either antibody before fixation. However, if these same sections were incubated with additional anti-FLAP or anti–5-LO after sectioning the nuclear envelope was clearly labeled. When interpreting these results it must be noted that the anti-FLAP antibody used in these experiments was an anti–peptide antibody that recognizes a discrete region of the FLAP amino acid sequence. Our results clearly show that this region of FLAP is oriented toward the lumen of the nuclear envelope. Thus, unless the protein reverses its orientation through the lipid bilayer, it would be expected that if FLAP were present in the plasma membrane the antibody binding domain should be exposed to the extracellular medium and thus be available to bind externally applied antibody before fixation and subsequent sectioning. This consideration is not a factor in interpreting the results with anti–5-LO since this probe was a polyclonal antibody that would be expected to recognize multiple epitopes at various random locations on the protein. These data thus demonstrate that FLAP and 5-LO are not detectable on the plasma membrane, and also suggest that fixation does not interfere with the ability of the anti-FLAP antibodies used in our experiments to bind FLAP.

The observation that the nuclear envelope is the intracellular site at which 5-LO and FLAP act is consistent with the observed correlation between the potency of 5-LO and FLAP inhibitors and their lipophilicity (43). MK-886, a first generation leukotriene synthesis inhibitor that binds to FLAP, is lipophilic and has been shown to partially block increased urinary LTE_4 excretion after antigen challenge in asthmatics, and to partially inhibit antigen-induced bronchoconstriction (44). MK-591, a more intrinsically potent FLAP inhibitor is also lipophilic, and has been shown to completely inhibit LTB_4 production in whole blood assays and urinary LTE_4 production, after a single dose in normal human volunteers

(45). These observations suggest that lipophilicity, as it relates to the capacity of potential leukotriene inhibitors to penetrate cell membranes and thereby gain access to the nuclear envelope, should be a major consideration in the design of potential therapeutic agents.

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